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TITLE: CHEK2*1100delC Variant and BRCA1/2-Negative Familial Breast Cancer - A Family-Based Genetic Association Study

PRINCIPAL INVESTIGATOR: Habibul Ahsan, M.D.

CONTRACTING ORGANIZATION: Columbia University
                                            New York, NY 10032, USA

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14. ABSTRACT
We propose to examine the association between the CHEK2*1100delC gene variant and breast cancer among BRCA1/2-negative families. Vital to DNA replication and normal growth of breast cells (like all other cells in the body) is their ability to detect aberrations/damage in the DNA, and subsequently to halt the replication process, correct errors if possible, and either resume normal cell replication or initiate cell death. The CHEK2 gene, the human ortholog of yeast CDS1 and Rad53, encodes a cell-cycle checkpoint kinase that plays a role in DNA repair processes involving BRCA1 and p53 and is thus a candidate gene for familial breast cancer and Li-Fraumeni Syndromes (LFS). The proposed study, by examining CHEK2 in familial breast cancer, will provide additional knowledge to enhance our understanding of the role of CHEK2 gene in breast cancer. By estimating the absolute and relative risk of breast cancer in relation to the CHEK2*1100delC variant, the proposed study will offer direct evidence on assessing genetic risk of familial breast cancer.

15. SUBJECT TERMS
Epidemiology, Genetics, Etiology, Genetic Epidemiology

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INTRODUCTION:

The human checkpoint kinase 2 (CHEK2, also known as CHK2), the human ortholog of yeast Cds1 and Rad53, encodes a cell-cycle checkpoint kinase that plays a role in DNA repair processes involving BRCA1 and p53 and is thus a candidate gene for familial breast cancer and Li-Fraumeni Syndromes (LFS)[1]. While several germline missense mutations in the CHEK2 gene have been reported in LFS families who are negative for p53 mutations, they were not associated with breast cancer[2;3]. One particular deletion mutation (1100delC) in the CHEK2 gene, which was first identified in a p53-wildtype LFS family with 4 breast cancers[2], has recently been shown to increase the risk of breast cancer in North American and European families who are negative for BRCA1/2 mutations as compared to controls from the same countries[4]. This frameshift mutation on codon 366 (due to deletion of a single base), which causes premature termination at codon 381, clearly abrogates the kinase activity of the encoded protein[2;3]. We hypothesize that the CHEK2*1100delC allele is associated with breast cancer in families who do not harbor mutations in the BRCA1/2 genes. Using a family-based design, we propose to examine this hypothesis among 1,612 BRCA1/2 negative families from 5 North American centers participating in the NCI's Breast Cancer Family Registry (BCFR) for whom genomic DNA and questionnaire and family data have already been collected.

BODY:

This is the final report of the study. We have aimed to finish tasks 4-6 listed below during the last year in the approved Statement of Work. To date, the majority of tasks for years 1-3 have been accomplished and are described below. Within the last year we also genotyped additional family members and subjects from some of the BCFR sites that were not included in the previous genotyping. This additional typing allows us to estimate unbiased penetrance for the mutation. We currently are in the process of data analyses and manuscript writing.

Eligible Families for the Study

All eligible families were ascertained. Families that were eligible for this study had a) at least two first or second degree relatives who are affected with breast cancer (where at least one was diagnosed before age 50), OR b) one breast cancer diagnosed before age 40, OR c) a male breast cancer. Eligible families must have had genomic DNA either for at least two first degree family members (where at least one affected with breast cancer) OR at least two second degree family members (where both affected with breast cancer).

Subjects Recruitment and Data/Sample Collection

All relevant questionnaire data and genomic DNA were obtained. Questionnaire data on subjects from all centers are maintained in a centralized database by the BCFR Informatics Core at University of California – Irvine (http://www.cfr.epi.uci.edu). All data relevant for this study was electronically transferred to Columbia University. For CHEK2*1100delC genotyping, we obtained stored samples of genomic DNA from eligible participants. Genomic DNA for the eligible participants was shipped in 0.5 µg aliquots to Columbia University for CHEK2*1100delC detection.

For the study, a separate database specific for this project has been created containing all relevant data. Breast cancer cases and familial controls with available blood samples were included in the proposed study. Standard data cleaning and editing have been performed on these data to ensure the case-control status.

Laboratory Assay

Genotyping for the CHEK2*1100delC mutation in all DNA samples was performed in Dr. Ahsan's laboratory at Columbia University. A two-stage PCR was used to specifically amplify CHEK2 exon 10 from chromosome 22 only. The primary PCR primers were: Forward: ATCTGAGGGTCATGCCTGCCTTTCTGTGTAG, Reverse: GTACATCAGTGACTGTGAAAAAGCAATTATTC. The PCR conditions per reaction: 2.5 µl 10X Expand Taq Long Template PCR system buffer, Boehringer Mannheim Buffer, 1.25 µl 2.5mM dNTP, 0.625 µl 20mM forward primer, 0.625 µl 20mM reverse primer, 17.75 µl H2O, 0.25 µl Taq/Pol Expand Taq Long Template PCR system, (preincubated with TaqStart Antibody, Clontech) and 2 µl genomic DNA (5 ng/ µl). Cycling conditions

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were: 95°C for 2min followed by 30cycles of 95°C for 1 min; 52°C for 1 min; 72°C, 10 min followed by 72°C for 5 min. The secondary primers were

GTACAATAGAAACTGATCTAGCCTACG and CTGGACAACAGAGCAAGACACATTTG. For the secondary reaction conditions were: 5 µl 10X Buffer, 1.0 µl 2.5mM dNTP, 0.5 µl 20mM forward

primer, 0.5 µl 20mM reverse primer, 35.5 µl H2O, 0.5 µl Taq/Pol and 7 µl of 10-2 dilution of primary PCR. Cycling conditions were 95°C for 5min followed by 35 cycles of 95°C for 30sec; 54°C for 30sec; 72°C for 45s followed by 72°C for 5min.

The single base deletion variant CHEK2*1100delC was detected in the amplified product using single nucleotide extension using fluorescence polarization to detect incorporated nucleotides. The wild type sequence at the deletion site is TACTGAT while that of the variant is TATGAT. After the second step PCR, primers and dNTPs were digested with 1 unit of shrimp alkaline phosphatase (1 µg/µl, Roche) after addition of 1µl of 10x buffer and 1 unit E.Coli exonuclease I (10 µg/µl, United States Biochemical, Cleveland, OH) and 7.9 µl of water for 45 min at 37o followed by heating at 95o for 15 min. Single base extension reaction was performed using Acycloprime FP SNP Detection kit containing the appropriate ddNTPs labelled either with R110 or TAMRA (Perkin Elmer Life Sciences, Boston MA). To 7 µl of reaction mixture was added 0.05 µl Acycloprimer enzyme, 1 µl Terminatior mix, 2 µl 10x reaction buffer, 0.5 µl extension primer (10 pmol/µl) and 9.45 µl water. Extension was carried out by heating at 95o for 2 min followed by 30 cycles of 95o for 15 sec and 55o for 30 sec. Plates were read on a Perkin Elmer Victor fluorescent reader. The wildtype and variant alleles had different base incorporation causing differences in the fluorescence polarization, which was the basis for genotyping. In addition to assay specific quality control samples, 10% of samples were reassayed after relabeling to keep laboratory personnel blinded to identity.
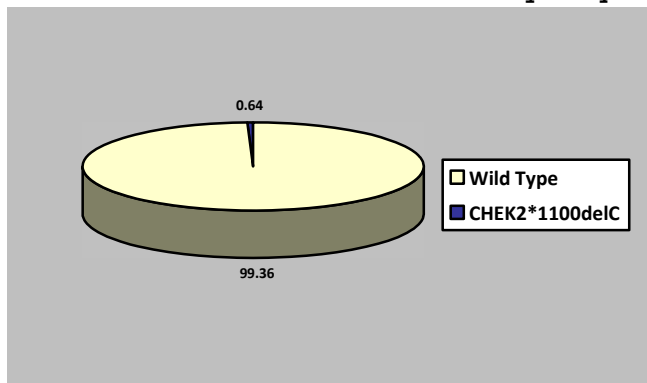
DNA samples of 4093 individuals were genotyped to date for the CHEK2 single base deletion (1100delC) variant by a two-step PCR (to specifically amplify CHEK2 exon 10 on chromosome 22) followed by single nucleotide extension on the amplified product using fluorescence polarization to detect incorporated nucleotide for the CHEK2 gene.

Formal statistical analyses for penetrance and effects of mutation on breast cancer are in progress. Preliminary genotyping results to date are summarized in Table 1 below.

Table 1. CHEK2*1100delC Allele Genotyping Results for a Total of 4,093 Women from USA (Metropolitan New York and Northern California) and Canada (Ontario)

No. of DNA samples genotyped	4093
No. blank or no call	210
No. wild type	3858
No. with CHEK2*1100delC mutation	25
CHEK2*1100delC mutation prevalence	0.64
Genotyping call rate	94.9%

Figure 1. CHEK2*1100delC Mutation Frequency based on 3883 Genotyped Samples

KEY RESEARCH ACCOMPLISHMENTS:
Year 1

We identified eligible families and reviewed all pedigrees to confirm eligibility based on family history, DNA availability, BRCA mutation status and other criteria.

All participating centers had to perform database search for providing relevant family, questionnaire, biospecimen and pedigree data.

We requested and obtained DNA samples and questionnaire data for eligible study participants from participating BCFR sites.

Participating sites had to re-extract DNA samples on many samples and needed to aliquot the samples based on a structured protocol.

Our laboratory at Columbia received all samples, checked for sample integrity, established laboratory database and inventory procedures.

Our laboratory conducted sample quality control and sample re-extraction and processing for all DNA samples.

We developed the laboratory mutation detection assay that involved several steps given the complexity of the assay because of the location of the mutation and presence of pseudogenes containing similar sequences. Development of a two-step PCR, associated primers and assay optimization parameters were required. Extensive piloting was done to finalize these aspects and successfully developing the assay.

We established quality control procedures by adding duplicate samples and also positive and negative controls in each runs.

The mutation detection assay was implemented using Fluorescent Polarization single-base extension assay with actual detection by a Tecan reader. The genotype calling algorithm was optimized to enhance the call rates.

Year 2

Our laboratory performed CHEK2*1100delC genotyping assay by performing two-step PCR and single nucleotide extension assay using fluorescent polarization on all 4,093 samples.

All detected mutations were confirmed by direct capillary sequencing of the parent DNA sample using ABI instrument at the DNA sequencing core facility at Columbia.

Year 3

All raw genotyping data were cleaned and edited for completeness, consistency and errors.

All laboratory data were merged with questionnaire and family data and transferred to Dr. Alice Whitemore at Stanford University for statistical analyses.

At the time of writing this report, statistical analyses for assessing mutation prevalence and penetrance have began based on modified segregation and other relevant statistical genetics methods, as appropriate for the study hypotheses.

REPORTABLE OUTCOMES:
    Kibriya M, Chen Y, Senie R, Andrulis I, John E, Daly M, Buys S, Whittemore A, Santella R, and Ahsan H. CHEK*21100DELC variant and BRCA1/2-negative familial breast cancer – a family-based genetic association study. Presented at the fourth Era of Hope meeting for the Department of Defense (DOD) Breast Cancer Research Program (BCRP), June, 2005, Philadelphia, Pennsylvania.

Kibriya M, Chen Y, Senie R, Andrulis I, John E, Daly M, Buys S, Whittemore A, Santella R, and Ahsan H. CHEK*21100DELC variant and BRCA1/2-negative familial breast cancer – a family-based genetic association study. To be presented at the seventh Era of Hope meeting for the Department of Defense (DOD) Breast Cancer Research Program (BCRP), June, 2008, Philadelphia, Pennsylvania.

CONCLUSIONS:
We have accomplished most of the tasks in the approved Statement of Work for years 1-3 as planned. We are in the process of data analysis and manuscript preparation.

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APPENDICES:
Not applicable.